In vitro basis for schedule-dependent interaction between gemcitabine and topoisomerase-targeted drugs in the treatment of colorectal cancer

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Background: While combination of gemcitabine with anti-topoisomerase poisons is routinely used in oncology, little is known on the biological interactions between these drugs.

Design: To understand the cellular basis for this association, we hypothesized an interaction of the two agents at the topoisomerase level. A real-time RT-PCR method was designed to quantify topoisomerase expression after treatment with gemcitabine (GEM) in two human colon adenocarcinoma cell lines. Efficacy of drugs as single agents and in combination was analyzed on the basis of their cytotoxic effects.

Results: We showed that a) gemcitabine induces expression of all major eukaryotic topoisomerases (I, II α and β) at definite times after drug administration; b) cytotoxicity was more relevant when cells were treated with GEM and the topoisomerase poison within a short period of time. In particular synergistic effects were found when the anti-topoisomerase II agent was given 3 h after gemcitabine or when the anti-topoisomerase I drug was delivered 3 h before or after the antimetabolite.

Conclusions: These findings help explaining the effectiveness of the combined therapy GEM/topoisomerase poisons and suggest a drug administration protocol for clinical treatment.

Key words: antimetabolite, colorectal cancer, cytotoxicity, real time RT-PCR, synergism, topoisomerase poison
synchronized combinations of the test drugs were measured. We were able to show that (i) GEM induces expression of all topoisomerase enzymes at definite times after drug administration; (ii) cytotoxicity was more relevant when cells were treated with GEM and the topoisomerase poison within a short period of time, and, in general, when the topoisomerase-targeted drug followed GEM.

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**materials and methods**

**drugs and cell lines**

Gemcitabine was provided by Eli Lilly Italia, Topotecan by Smyrkline Beecham and Etoposide by Bristol Mayer Squibb. Further dilutions were made in DMEM supplemented with heat inactivated 10% fetal bovine serum (FBS), 10 μM glutamine, penicillin (100U/ml) and streptomycin (100 μg/ml). Human colon adenocarcinoma cell lines LoVo and HT-29 (American Type Culture collection, ATCC, Rockville, Maryland) were used. Cells were routinely cultured in DMEM, 10% FBS, 10 μM glutamine, penicillin (100U/ml) and streptomycin (100 μg/ml) and kept at 37°C-5% CO2.

**cytotoxicity**

1.5 × 10^5 LoVo or HT-29 cells/well were plated in 96 well plates in a total volume of 200 μl. The seeding cell concentration was such as to allow a logarithmic growth. After 24 h GEM was added at increasing concentrations (0.01, 0.1, 1, 10 and 100 μM). After 96 h, cell viability was evaluated by a standard MTT assay. Briefly, 10 μl/well of MTT solution (4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) were added to cells. After 3–4 h medium was removed and formazan crystals were solubilized with 200 μl of HCl 0.01 M/10% SDS. Absorbance was measured at 550–570 nm. Each drug treatment was repeated six times. Cytotoxicity was defined as the drug concentration able to induce death in 50% of the cellular population (IC50). For drug combination cytotoxicity assay, increasing concentrations of topotecan or etoposide (0.01–10 μM) were administered to LoVo cells 3 h before, 3 h or 24 h after GEM (0.5 μM). After 96 h, cell viability was evaluated by a MTT assay, as described above. Each drug combination was repeated three times.

**isolation of RNA and real-time RT-PCR analysis**

Total RNA was isolated from LoVo or HT-29 cells following a single step acid guanidium phenol-chloroform extraction procedure employing OMNIzol (Euroclone, UK). Real-time quantitative RT-PCR analysis was performed on a Light-Cycler instrument (Roche). Real time RT-PCR analysis was performed in six well plates. 8 × 10^5 cells were plated per well. After 24-h incubation, LoVo or HT-29 cells were treated with GEM (0.5 or 0.06 μM, respectively). Cells were harvested at 2, 3, 6 and 24 h time intervals after drug treatment and total RNA was isolated using OMNIzol; cDNA was synthesized from 3 μg of mRNA with MuLV reverse transcriptase in the presence of random hexamers.

**results**

**GEM is differently effective on two human colon adenocarcinoma-derived cell lines**

The effect of GEM as a single agent was evaluated on two different cell lines derived from human colon adenocarcinoma, LoVo and HT-29. The efficacy of GEM as antiproliferative agent was measured by its ability to induce cell death. Both cell lines were treated with increasing amounts of GEM (0.01 μM–100 μM), and after 96 h cellular viability was assessed by a standard MTT assay. The results show that GEM was differently effective on the two cell lines (Figure 1). In particular, IC50 values corresponded to 0.5 μM and 0.06 μM for LoVo and HT-29 cells, respectively.

**GEM induces expression of topoisomerases enzymes in vitro 3 h after treatment**

To understand the basis of antimitabolite/topoisomerase poison drug association we measured the effects of GEM on

![Figure 1. Cytotoxicity of GEM as a single agent. Percentages of cell viability of LoVo (●) and HT-29 (■) cells after treatment with increasing concentrations of GEM measured by MTT assay.](image-url)
topoisomerases expression in human colon adenocarcinoma cell lines. Topoisomerase I, II α and II β expression was measured by means of mRNA levels, which were quantified by real-time RT-PCR (see Materials and methods). The GAPDH gene was used as an internal standard to normalize data. As shown in Figure 2, topological enzyme levels indeed increased after exposition to a constant amount of GEM (0.5 μM in LoVo and 0.06 μM in HT-29, which corresponded to IC₅₀ values in the two cell lines, respectively). In particular, topoisomerase expression was considerably higher in LoVo cells as compared to HT-29. In LoVo cell line, enzyme expression was constantly increased in a time range of 2–3 h after treatment with GEM. At 6 h topoisomerase levels returned to almost basal intensity, hence all data were acquired within 6 h. Topoisomerase I was the most remarkably induced: after 2 h levels were 5 times as compared to basal expression (Figure 2A). Topoisomerase II α and β shared a similar degree of induction (2.5 times over basal values) (Figure 2B and C). In the HT-29 cell line, induction of expression was less relevant; nonetheless both topoisomerase I and II α were as well increased after 2–3 h of cell exposition to GEM (128% and 151%, respectively) (Figure 2A and B) and topoisomerase II β showed induction after 6 h (139%) (Figure 2C). Increased expression over basal levels was exhausted within 6 to 24 h.

**schedule dictates efficacy of antimitabolite/topoisomerase poison combination**

To determine optimal administration schedule of antimitabolite/topoisomerase poison combination, cells were treated with a constant amount of GEM (0.5 μM) and topotecan or, alternatively, etoposide at increasing concentrations (0.01–10 μM), based on three different protocols: 1) topoisomerase poison given 3 h before GEM; 2) topoisomerase poison given 3 h after GEM; 3) topoisomerase poison given 24 h after GEM. Cell viability was measured 96 h after treatment by a MTT assay. Measurements were conducted on LoVo cells since they displayed the highest GEM-induced topoisomerase expression. Results of drug combination were analyzed based on the activity of the topoisomerase poison alone and on the theoretical effect of GEM associated to the anti-topoisomerase compound, calculated as the algebraic sum of the effects of each drug separately. According to the fractional effect analysis, a correspondence of the theoretical and experimental curves would reveal additive outcomes, while experimental curves below or above the theoretical curve would indicate synergistic or antagonistic effects, respectively [8] (Figure 3). Results represented an average of three different experiments for each drug combination. Differences between theoretical and experimental curves proved significant, being standard deviations less than 20% at each point. In the case of the topoisomerase I poison, additive effects were found at topotecan concentrations of 1 and 10 μM for the three different protocols (Figure 3A–C). However, at lower concentrations (0.01 and 0.1 μM), the behavior was clearly biased: synergistic when topotecan was administered 3 h before or after GEM, antagonistic when the anti-topoisomerase was administered 24 h after GEM (Figure 3A–C). In the case of the topoisomerase II agent, the effect of drug combination more evidently depended upon administration protocols: when etoposide was given 3 h before or 24 h after GEM, the outcome was antagonist, while when the anti-topoisomerase was given 3 h after GEM, the results were synergistic at lower etoposide concentration (0.01 and 0.1 μM) and additive at higher amounts (1 and 10 μM) (Figure 4D–F). To note that the synergistic effects, when the anti-topoisomerase drug was given 3 h after GEM, were slightly improved for topotecan compared to etoposide.

**discussion**

While combination of GEM with anti-topoisomerase poisons is routinely used in the clinical treatment of several types of cancers [7, 9], little is known on the biological interactions between these drugs. To understand the mechanistic basis for this association, we hypothesized an interaction of the two agents at the topoisomerase level. We first measured the IC₅₀ values of GEM in two different human colon adenocarcinoma derived cell lines. GEM resulted more effective on LoVo than HT-29 cells, possibly due to the faster replication rate of the
formers. IC50 values were next used as reference concentrations at which cells were treated for the subsequent measure of topoisomerase expression. All the principal eukaryotic topological enzymes were taken into consideration. In fact each has a peculiar role in the genomic DNA processing [11]. We found that treatment of both cell lines with the antimetabolite drug induced overexpression of all the topological enzymes. Interestingly, in all cases expression was maximal within 3 h from treatment with GEM and returned to basal levels within 6–24 h. Likely because of their faster growth, LoVo cells were more susceptible to GEM-mediated topoisomerase induction; in particular, topoisomerase I resulted expressed two times more than the topoisomerases II. Conversely, in HT-29 cells all three enzymes were similarly induced, with a slight preference for topoisomerase IIα and β.

Based on the above results, we hypothesized that the highest cytotoxic effect could be achieved when the topoisomerase poison was given in the time range of maximal GEM-mediated induction of the topological enzymes. Hence, cells were treated with a combination of GEM and anti-topoisomerase I or II drugs, given according to three different protocols. Indeed we found that when the topoisomerase poison was given 3 h after GEM, the effect for both drug associations was synergistic at lower anti-topoisomerase doses and additive at higher concentrations. Contrary, when topotecan or etoposide were given as far as 24 h after GEM, the effect was solidly antagonistic. However, if the topological poisons were administered 3 h before GEM, the effect was biased: synergistic/additive for topotecan and antagonistic for etoposide.

We propose that in the case of the topoisomerase II poison, cytotoxicity is exerted in part by GEM-mediated inhibition of DNA polymerase α [12], with termination of nascent DNA helixes or impairing of genome repair; in part by GEM-mediated induction of topoisomerase II, which increases double strand DNA cleavage that is subsequently stabilized by etoposide, resulting in massive DNA damage and cell death. This holds true in the case of the topoisomerase I poison, which stabilizes single-stranded DNA scission. However, the fact that treatment with the anti-topoisomerase I 3 h before or after GEM gave similar results, indicates that effectiveness of GEM/topotecan association is likely mediated by additional mechanisms. It is has been reported that topoisomerase I participates in RNA polymerases I, II, and III-mediated transcription, has a kinase activity, and binds directly to at least

Figure 3. Cytotoxicity of the topoisomerase poisons in combination with GEM (0.5 μM). LoVo cells were treated with increasing amounts of topotecan (A–C) or etoposide (D–F) separately or in combination with GEM. When in association, the topoisomerase poison was given 3 h before (A and D), 3 h after (B and E) or 24 h after GEM (C and F), as described on the right of the graphs. After 96 h cell survival was measured by a MTT assay. Cytotoxicity curves for the topoisomerase poison alone (●), and experimental (■) and theoretical (▲) cytotoxicity curves for drug combination are reported in each graph. Theoretical curves were calculated according to the fractional effect analysis [8]. A correspondence of the theoretical and experimental curves indicates additive outcomes, while experimental curves below and above the theoretical curve imply synergistic and antagonistic effects, respectively. The results represented an average of three different experiments for each drug combination. Standard deviations were in each case less than 20%.
two helicases, nucleolin and SV40 T antigen, acting as a multifunctional protein [13]. It is then possible that impairing of the ‘unconventional’ topoisomerase I activities induces genome instability, which is enhanced by the following treatment with the antimetabolite agent. Hence, in the case of the topoisomerase I poison, both topotecan and GEM would augment their activity reciprocally. This is in part substantiated by the slightly improved synergistic effect of the anti-topoisomerase I drug compared to that of etoposide.

Interestingly, higher synergistic effects can be noted using low topoisomerase poison concentrations. This fact can be reasonably ascribed to saturation in the number of cleavable complex units that can be generated following stimulation with a constant concentration of GEM. While further analysis will be performed in the near future to confirm these results both including other cell lines and using various antimetabolite /anti-topoisomerase agent combinations, the present findings help explaining the rational basis of the clinical GEM/topoisomerase poisons combination, and most importantly, give a rational indication of the most appropriate drug administration schedule in the clinic.

disclosures

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references